A Study on Real-Time Modification of the Refractive Index of A Surrounding Medium using a Uniform Microsphere in Digital Holographic Microscopy

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A study on real-time modification of the refractive index of a surrounding medium using a uniform microsphere in digital holographic microscopy

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ABSTRACT
An accurate refractive index of the surrounding medium is required to measure the accurate depth of the specimen in digital holographic microscopy (DHM). Many researchers have reported a method that uses blood smear to avoid overlapping of red blood cells (RBCs). Moreover, they have applied the refractive index of the blood plasma. However, the blood smear is not blood plasma, so that it cannot obtain the accurate depth information. In addition, it is difficult to measure the refractive index of the surrounding medium in every experiment. To solve this problem, in this paper, we propose a new method to record a hologram using a sample of a mixture of blood and a uniform microsphere. We have already known the size of the microsphere in the experiment. Thus, we can modify the refractive index of the surrounding medium. Finally, we can measure the accurate depth information of the specimen using the modified refractive index of the surrounding medium. The proposed method can be used not only in RBCs but also in the study of cells or microbial.

CCS Concepts
• Computing methodologies →Artificial intelligence → Computer vision →Image and video acquisition →3D imaging. • Computer systems organization →Architectures →Other architectures →Optical computing. • Applied computing →Life and medical sciences →Bioinformatics.

Keywords
Digital holography; Phase-contrast imaging; Biomedical imaging; Refractive Index;

1. INTRODUCTION
Holography was suggested by Dennis Gabor in 1948 [1] and has been studied by many researchers [1-17]. Moreover, digital holography (DH) was published by Joseph Goodman in 1967 [2]. And then, the first paper of digital holographic microscopy (DHM) was published in the middle of the 1990s [3, 4]. The advantage of DHM is that it can obtain three-dimensional (3D) information of an object using amplitude and phase, unlike a conventional microscope that only records intensity. For this reason, it has been applied to many applications, such as the diagnosis of diseases [5-7], 3D profiling of microstructure [8-10], refractive index measurement [11], and microbial research [12-14]. Recently, the diagnosis of diseases with statistical analysis of the depth information for red blood cells (RBCs) using DHM has been studied [5-7]. To obtain the accurate depth information, we need to know the exact refractive index of both the specimen and the surrounding medium [15-17]. This is because the refractive index difference between them is required when calculating the depth information using the phase difference from the recorded holograms [6, 12, 15-17]. In the DHM study for the analysis of RBCs, if a sample is prepared by dropping blood on a slide glass and covered with a cover glass, the refractive index of the surrounding medium can be used as a refractive index of the blood plasma. However, if the working distance of the objective lens is shorter than the thickness of the cover glass, it cannot accurately focus on the RBCs. Also, many kinds of research have used a blood smear sample to avoid the overlapping of RBCs. In this case, blood plasma is hardened by platelets. However, the refractive index of the hardened blood plasma is different from the refractive index of liquid blood plasma. Therefore, it is difficult to obtain the accurate depth information of RBCs. To solve this problem, we propose a new method to obtain the accurate depth information of RBCs by measuring the refractive index around the sample and mixing the

uniformed microspheres into the blood. It is expected that our method can be used not only in RBCs but also in the study of cells or microbial.

This paper is organized as follows. In section 2, we explain the principles of digital holography and the formulas to calculate the depth information of the specimen. Then, the experimental setup and conditions are described in section 3. Besides, we show the experimental result by our proposed method in section 4. Finally, we discuss the expected effectiveness of our method based on the experimental results in section 5.

2. THEORY

2.1 DIGITAL HOLOGRAPHY

Digital holography (DH) is a technique for obtaining depth information of an object by recording the amplitude and phase of light scattered from the object. However, because image sensors can only record intensity, phase information cannot be obtained. For this reason, DH records the interference pattern between the reference wave and the object wave using the image sensor. After that, we can calculate the phase from the recorded interference pattern. Finally, we can obtain the phase difference between the object wave and the reference wave.

The intensity of the interference pattern between the object wave and the reference wave on the image sensor is described as follows:

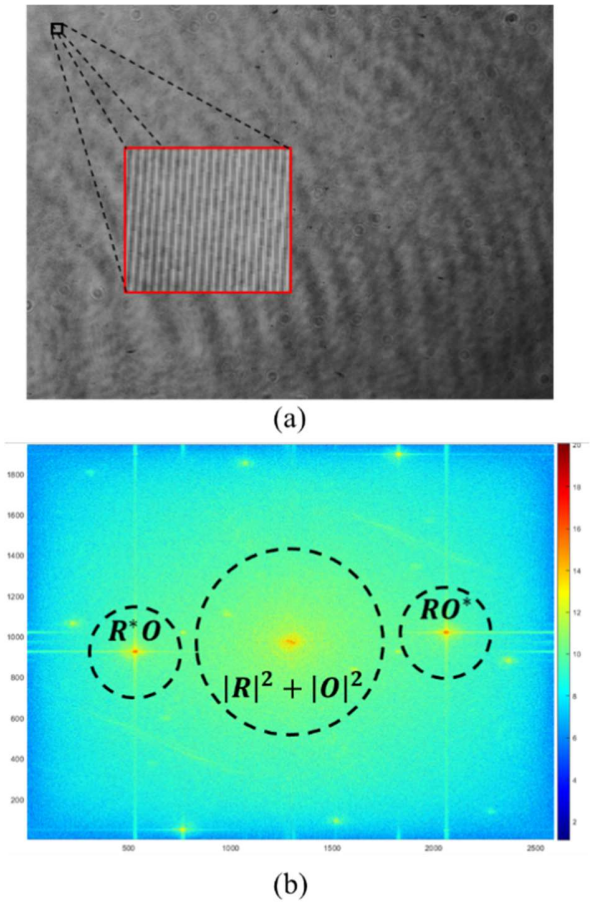


Figure 1. (a) Interference pattern between the reference wave and the object wave, (b) Fourier domain of the interference pattern.

$$I_{Hol} = |R|^2 + |O|^2 + R^*O + RO^*, \quad (1)$$

where I_{Hol} is the intensity of the recorded hologram by the image sensor, $|R|^2$ and $|O|^2$ are the intensity of the reference and the object wave, respectively. In addition, R^* and O^* denote the complex conjugates of the reference and the object wave, respectively [9, 18, 19].

Figure 1 shows the interference pattern of the reference wave and the object wave and its Fourier domain. The red square in Fig. 1(a) is a magnified image to observe the interference pattern. As shown in Fig. 1(b), $|R|^2 + |O|^2$ is the DC term, R^*O and RO^* represent the interference term in the Fourier domain, respectively [9, 18]. Also, real and twin images of the object are given by R^*O and RO^* in the Fourier domain [9]. For this reason, we crop the one of two terms from the Fourier domain, and then the cropped area has been shifted to the center of the Fourier domain. Finally, we can generate the phase of the image by inverse Fourier transform.

2.2 CALCULATING DEPTH INFORMATION USING PHASE DIFFERENCES IN DHM

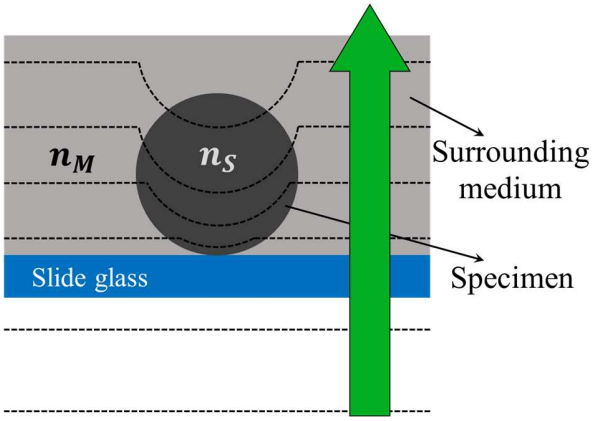


Figure 2. Example of the wavefront scattering in the specimen.

In Fig. 2, the black dashed line is the wavefront, and the green arrow is the direction of the wave. When the plane wavefront passes through the sample, the phase changes due to the refractive index difference between the specimen and the surrounding medium. Then, when there is no sample, the only reference wave is recorded to obtain the phase information, and the final phase difference is calculated by using both object and reference phase information. Finally, the depth information of the specimen can be calculated using this phase difference. This is expressed in mathematically as follows [12, 15-17]:

$$\phi_{obj}(x,y,t) - \phi_{ref}(x,y) = \Delta\phi(x,y,t)$$
$$= \frac{2\pi}{\lambda}(n_S - n_M)h(x,y,t), \quad (2)$$

where ϕ_{obj} and ϕ_{ref} are the phases of the object wave and the reference wave. In addition, λ is the wavelength of the illuminated light source, n_S and n_M are the refractive indices of the specimen and the surrounding medium, and h represents the height of the specimen, respectively. This equation can be simplified as follows:

$$h(x,y,t) = K\Delta\phi(x,y,t), \quad (3)$$

where constant K is $\lambda/(2\pi\Delta n)$ and Δn is the refractive index difference between object and reference. Therefore, we can obtain the depth information from the phase differences in DHM.

3. EXPERIMENTAL SETUP

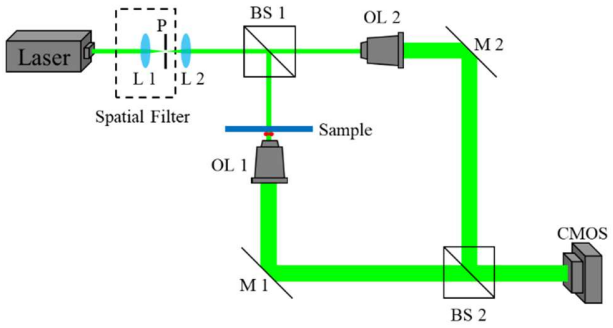


Figure 3. Experimental setup.

Figure 3 illustrates the experimental setup. L is the lens, P is the pinhole, M is the mirror, OL is the objective lens, and BS is the beam splitter. We use Mach–Zehnder interferometer with a spatial filter in front of a 532nm green color semi-conductor laser. After passing through the spatial filter, the laser beam passes through a collimating lens (L2) to create a plane wave, where the diameter of laser beam through L2 is 2 mm. The two objective lenses used in this experimental setup are 40x, and their working distance is 0.6mm. We use the acA2500-14uc model of Basler to capture the hologram. The specifications of this image sensor are summarized in Table 1.

Table 1. Specifications of the image sensor to capture the hologram

Company	Basler
Model	acA2500-14uc
Sensor	MT9P031
Sensor size	5.7 mm × 4.3 mm
Resolution (H×V)	2590 px × 1942 px
Pixel Size (H×V)	2.2 µm × 2.2 µm
Color	Mono/color
Frame rate	14 fps

In this experiment, since we are not interested in the color of the specimen, the sensor color setting is mono. In addition, the exposure time is 35µs. Then, healthy male human blood and 10µm polystyrene microspheres are mixed and placed on a slide glass.

4. EXPERIMENT RESULT

When recording the hologram by DHM, we focus on the widest point of the specimen to prevent the phase error. For this reason, when we capture the microspheres hologram, we have to focus on around 5µm from the bottom. Also, when we obtain the RBCs hologram, we have to focus on around 1µm from the bottom. This is because the height of the microspheres is expected around 10um, and the height of red blood cells is expected around 2 ~ 2.5µm. Therefore, we need different focus holograms to obtain the RBCs and microspheres height data, respectively.

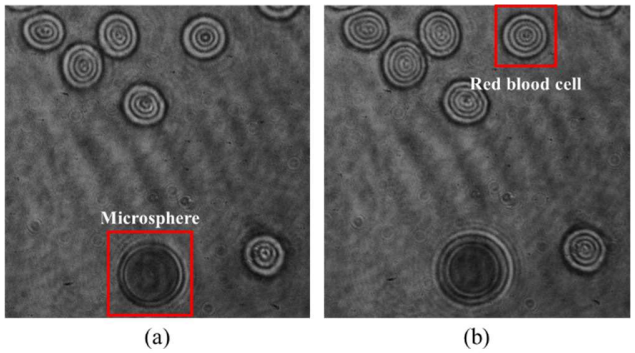


Figure 4. Recorded holograms using the proposed method. (a) Focus on the microsphere. (b) Focus on the RBCs

Figure 4 shows the recorded holograms using the proposed method. We can observe that there are RBCs and microspheres together in Fig. 4. To minimize DC noise, we set the spacing of the interference pattern as narrow as possible, so that the position of $\boldsymbol{RO}^*$ in the Fourier domain is far from the DC term. The material of the microsphere is polystyrene, and its refractive index for 532nm illuminated light source is 1.5983. However, the refractive index of the surrounding medium is unknown, so that applying the refractive index of blood plasma (1.34) to calculate the depth information. After that, we can modify the refractive index of the surrounding medium using the accurate depth information of the microsphere.

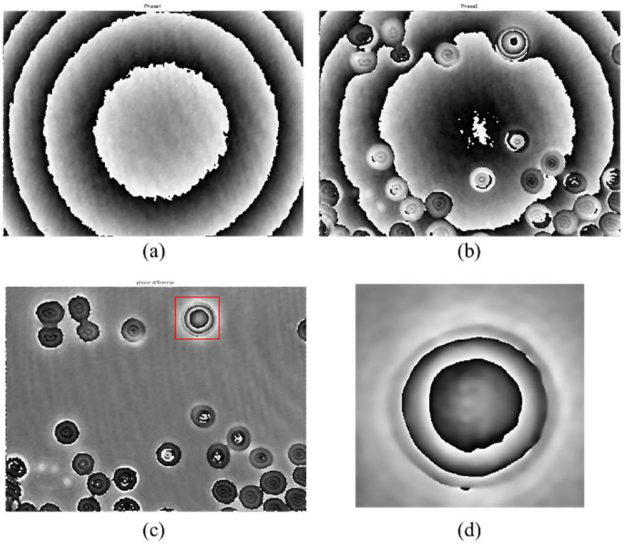


Figure 5. Calculation of phase differences using reference and object hologram, (a) and (b) Phase of the reference and the object holograms, (c) Phase differences between (a) and (b), (d) Region of the interest (red square) from (c).

Figure 5 shows how to calculate phase differences to obtain depth information of the specimen. Figure 5(a) is the phase of the reference hologram, and (b) is the phase of the object hologram focused on the microsphere. In addition, Fig. 5(c) is the phase difference between Fig. 5(a) and (b).

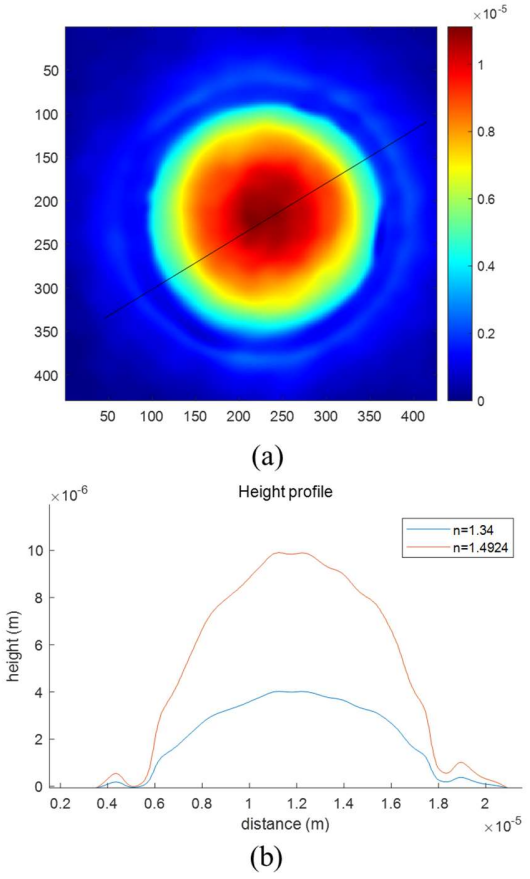
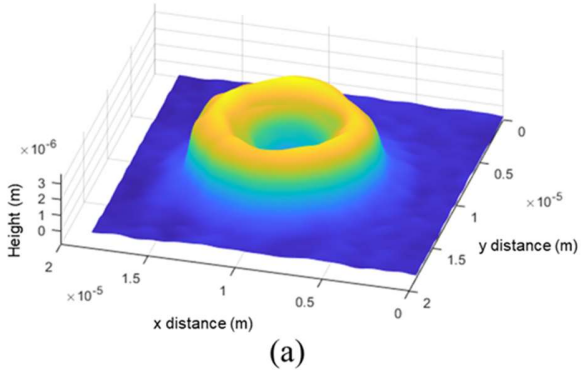


Figure 6. Depth profile of the microsphere. (a) 2D depth profile, (b) 1D depth profiles using the refractive index of the blood plasma (1.34) and modified refractive index (1.4924).

In this paper, we use a Goldstein phase-unwrapping algorithm [20]. Figure 6 shows the phase-unwrapped image of Fig. 5(d). Figure 6(a) shows a 2D depth profile of the microsphere. In addition, Fig. 6(b) shows a 1D depth profile of the black line from (a) using the refractive index of the blood plasma and modified refractive index. The blue graph in Fig 6(b) shows incorrect height of microspheres because the refractive index of the surrounding medium is not the same as the blood plasma. So, when we modify the refractive index based on the height information of the microsphere, the correct height of microspheres can be achieved as shown in Fig. 6(b). The modified refractive index of the surrounding medium is 1.4924.



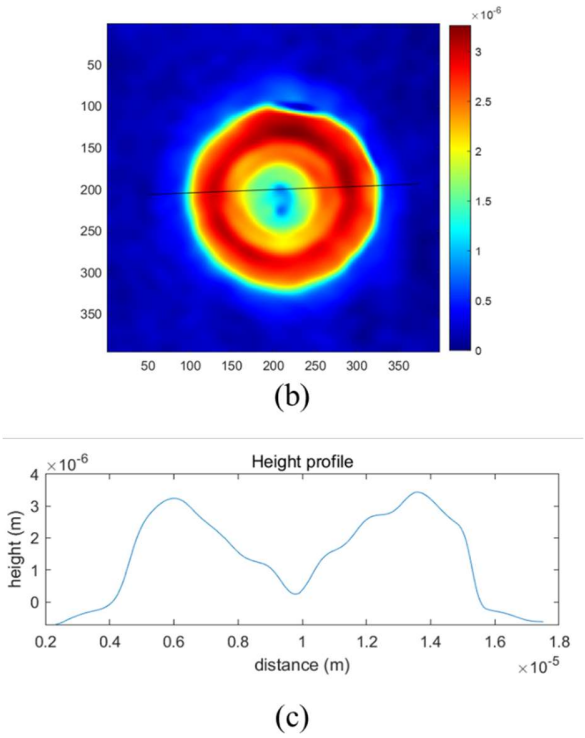


Figure 7. Depth profile of the RBC with modified refractive index of the surrounding medium (a) 3D depth profile, (b) 2D depth profile, (c) 1D depth profile of the black line in (b).

Figure 7 shows the depth profiles of the RBC with a modified refractive index (1.4924) of the surrounding medium. In this experiment, the maximum height of RBC is around 3μm. This is slightly larger than the average RBC size.

5. DISCUSSION

In this paper, we have proposed a DHM method of mixing microspheres with a specimen to find the accurate refractive index of the surrounding medium in real-time. First of all, the depth information of the microsphere has been calculated using the expected refractive index of the surrounding medium, and the refractive index has been corrected by the height of the microspheres. And then, the depth information of the specimen has been calculated using the modified refractive index. As a result, the height of red blood cells obtained in this paper is up to 3μm, and the average height of typical red blood cells is 2 to 2.5μm. The refractive index applied in this paper may not be an exact value because the refractive index of RBCs is determined by the concentration of hemoglobin and the wavelength of the illuminated light source [21]. In addition, the modified refractive index may not be accurate because the height of the microsphere is not exactly 10μm. However, the proposed method has an advantage that the refractive index can be modified in real-time when acquiring depth information of a specimen which has the unknown refractive index of the surrounding medium or non-constant refractive index. For this reason, it can be applied to the study of microorganisms and RBCs using blood smear.

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